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DOI: <https://doi.org/10.1117/12.2002953>

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ZORA URL: <https://doi.org/10.5167/uzh-90585>

Conference or Workshop Item

Published Version

Originally published at:

Sobek, Jens; Schauer, Stefan; Rehrauer, Hubert; Fischer, David; Patrignani, Andrea; Landgraf, Stephan; Schlapbach, Ralph (2013). Single molecule interactions studied by using a modified DNA sequencer: a comparison with surface plasmon resonance. In: SPIE, San Francisco, 2 February 2013. SPIE - International Society for Optical Engineering, 85960Y.

DOI: <https://doi.org/10.1117/12.2002953>

Single-molecule interactions studied by using a modified DNA sequencer: A comparison with surface plasmon resonance data

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ABSTRACT

In this study we established the principles for using a commercial off-the-shelf DNA sequencer (RS, Pacific Biosciences) to measure biomolecular interactions.^{1,2} Binding between single oligonucleotides immobilized at the bottom of a nanowell to a complementary DNA strand was used as a model system. The influence of the labeling dye on the hybridization of a 7mer oligonucleotide was investigated. The resulting association and dissociation rate constants (k_{on} and k_{off}) and the calculated dissociation constants (K_d) were compared to data obtained from surface plasmon resonance (SPR) measurements. In the present study we identified a good agreement of the determined kinetic constants by the two methods investigated.

Keywords: Single-molecule study, zero-mode waveguide technology, fluorescence, hybridization, surface plasmon resonance, biomolecular interaction, short oligonucleotides, dye effect

INTRODUCTION

Determination of kinetic rate constants of molecular interactions is a central analytical task in bioanalytics. While SPR has been established as the method of choice in the recent past,^{3,4} it suffers from a number of limitations that are hard to overcome with the respective method. In order to broaden the analytical options for analyzing molecular interaction kinetics, we carried out the present study, in which we investigated the option to determine kinetic rate constants by single-molecule real-time (SMRT) measurements using a commercial DNA sequencer (Pacific Biosciences RS).² Further, we compared the results with data obtained by using SPR.

The underlying measuring principles of the two methods are very different. SPR is a label-free method performed in a microfluidic flow cell. Polarized light is used to excite surface plasmons to detect changes in refractive index in the vicinity of the surface caused by mass differences. The sensitive range extends to about 100 nm from the thin gold surface that is coated with a three dimensional dextran matrix to allow immobilization of the probe. Usually, 10^9 - 10^{12} probe molecules are immobilized for binding experiments in a flow cell.

In contrast, SMRT measurement in the RS is based on fluorescence detection of single molecules in a zero-mode waveguide (zmv) of 100 nm diameter that confines the reaction volume to about 100 zeptoliter. A SMRT chip is a nanostructure array made of 160000 zmw's that are used in parallel. Details of the technology are given in the literature.^{1,5} The SMRT chip surface is coated with a polyphosphonate modified with polyethylene glycol bearing biotin groups.⁶ The detection volume extends over only some 10 nm into the solution. The technology requires the use of fluorescent labeling dyes absorbing at the excitation laser wavelength available (532nm, 633 nm).

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MATERIALS AND METHODS

Materials

All measurements were performed in a HEPES based buffer (HBS, 150 mM sodium chloride, 10 mM HEPES, 3 mM EDTA, 0.05% v/v Tween-20, GE Healthcare, Uppsala, Sweden). Ultra pure and filtered water ("MilliQ", Millipore, Billerica, USA) was used for preparation of all solutions. Carboxymethyl dextran coated sensor chips CM5 and CMD-500L (CMD) were obtained from GE Healthcare (Uppsala, Sweden) and XanTec bioanalytics (Düsseldorf, Germany), respectively. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid, N-hydroxysuccinimid, and streptavidin were from Sigma (Buchs, Switzerland). Neutravidin was received from Thermo Scientific (Wohlen, Switzerland). HPLC purified oligonucleotides were from Microsynth (Balgach, Switzerland) and used as received. The sequences were designed for non-self-complementarity and absence of secondary structures. Oligonucleotide sequence data are given in Table 1.

Table 1. Sequence data of oligonucleotides.

Oligonucleotide	Sequence
7mer	5' CAGTGCC
CY3-7	5' CY3-CAGTGCC
DY547-7	5' DY-547-CAGTGCC
Bio34	5' Biotin-TTTTTGGAACTGTATT GGCACTG AGTAGACTCC

The probe is a 34mer 5'-biotinylated oligonucleotide (Bio34). The analytes are 7-mer oligonucleotides 5'-labeled with CY3 (CY3-7) and DY-547 (DY547-7), respectively. For synthesis of the 5-CY3-7mer, a phosphoramidite building block was used to incorporate CY3 dye at the 5'-end of the oligonucleotide (10-5913, Glen Research, Sterling, VA). DY-547 dye was added to a 5'-amine linker modified oligonucleotide as NHS ester (Dyomics, Jena, Germany). Structurally, the dyes only differ by the presence of two sulfonic acid groups in DY-547 introducing a net negative charge (-1) in contrast to CY3 that is positively charged (+1) (Figure 1), rendering DY-547 more hydrophilic. It has to be noted that the term CY3 is sometimes used for a dye containing two sulfonic acid groups, which in contrast to this, is DY-547 in this publication.

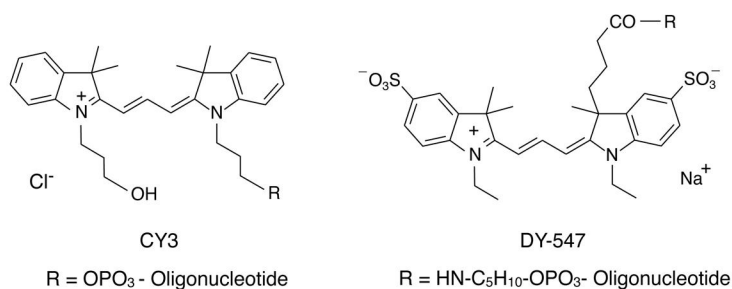


Figure 1. Molecular structures of the oligonucleotide-dye conjugates. For oligonucleotide sequence information see Table 1. In the free dye DY-547 is $\text{R} = \text{OH}$.

Absorbance and fluorescence measurements

Absorbance spectra of solutions were recorded with an Ultrospec 3300 pro UV/visible spectrophotometer (GE Healthcare, Glattbrugg, Switzerland) in 1 cm quartz glass cuvettes. Fluorescence was detected using a Safire2 fluorescence plate reader in top detection mode. Oligonucleotide solutions in HBS (100 nM, 1 μM) were excited at 500 nm with a bandwidth of 10 nm, both for excitation and detection. For comparison, DY-547 dye (free acid) was used (Dyomics, Jena, Germany).

Surface Plasmon Resonance

The optical evanescent wave sensor Biacore T100 (GE Healthcare, Freiburg, Germany) was used for the SPR measurements. The principles of this sensor have been described elsewhere.^{7,8} In brief, the binding of one molecule in

solution to an biotinylated other attached to a streptavidin coated carboxymethyl dextran matrix, itself being linked to the gold sensor surface, causes a change in refractive index. This change is detected by the sensor provided it takes place within the evanescent field which extends a few hundreds nanometers from the sensor surface. Data were collected with the biosensor instrument thermostated to 20°C. The SPR sensor is routinely cleaned to maintain the performance of the instrument using the “desorb” function in the Biacore Control Software. To prepare the sensor chip for interaction analysis, a conditioning solution was injected over the chip surface. A new sensor chip was inserted into the instrument and the system primed with HBS. The conditioning solution (50 mM NaOH, 1 M NaCl) was used to hydrate and clean the dextran layer and ensure minimum baseline drift. Three aliquots of conditioning solution were injected over all four flow cells for 60 s at a flow rate of 100 $\mu\text{L}/\text{min}$. The carboxymethyl dextran coated chip surface was modified to allow immobilization of a biotinylated probe. First, surface carboxy groups were activated using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide following the standard protocol. In a second step, the surface was coated with 50 $\mu\text{g}/\text{mL}$ streptavidin (CM5 chip) or neutravidin (CMD chip) in acetate buffer at pH 5. For probe immobilization, Bio34 was diluted to a final concentration of 2 $\mu\text{g}/\text{mL}$ in HBS and injected at a flow rate of 5 $\mu\text{L}/\text{min}$. A total of 280 (180) resonance units of Bio34 were thus immobilized on the CM5 (CMD) sensor chip surface. An initial series of five buffer blanks (start-ups) was injected first before each analyte series to fully equilibrate the system. The analyte samples were analyzed first from the lowest to the highest concentration and then run in duplicate from the highest to the lowest concentration. During each binding cycle the respective analyte was injected for 60 s at a flow rate of 30 $\mu\text{L}/\text{min}$ and dissociation was monitored for 300 s. The 7mer was typically measured in concentrations from 9.8 to 2500 nM, the CY3-7 and DY547-7 in concentrations from 9.8 to 1250 nM, all in two fold steps. Data sets were processed and analyzed using the Biacore T100 Evaluation Software version 2.0.3. Data were zeroed on the x- and y-axes at the time point of injection. The resulting sensograms were corrected for systematic artifacts, composed of drift, unspecific binding, signal jumps due to the injection needle positioning and bulk refractive index changes by subtracting the signal from a blank reference flow cell where no Bio34 was immobilized. This first referencing step significantly improved the quality of the binding surface data but did not consider the inherent systematic differences between the flow cells. Therefore, in a second step the average of the running buffer data from all binding events collected under the same conditions were subtracted from the sensograms.⁹ This led to high quality binding data that AB) including a term for mass transfer.¹⁰ Results from experiments using CM5 chips coated with streptavidin and CMD coated with neutravidin were very similar.

Single-molecule real-time measurements

Single-molecule measurements were performed in a commercial DNA sequencer (RS, Pacific Biosciences, Menlo Park, CA) converted by the company to enable manual experiments. The operating principle is described in Korlach et al.⁵ The surface of the SMRT cell is coated with a polyphosphonate functionalized with a biotinylated PEG. Based on this coating, the surface was prepared as follows for single-molecule hybridization: First, the cell surface was coated with 0.1 mg/mL neutravidin in HBS for 5 min. In a second step, a 5'-biotinylated 34mer oligonucleotide probe, 75 pM in HBS, was immobilized for 3 min. Finally, the analyte solution of CY3- and DY-547-labeled oligonucleotide, respectively, in HBS containing the triplet state quencher Trolox¹¹ and an oxygen quenching system based on protocatechuic acid/protocatechuate-3,4-dioxygenase¹² was added immediately before inserting the chip into the RS. There is a 20 min delay time before the measurement actually starts, needed by the RS for preparation including chip alignment. Dye fluorescence was excited at 532 nm and monitored in 80000 zmw's over a period of 5 to 15 min at 20°C. Measurements were performed at oligonucleotide concentrations between 40 and 100 nM. SMRT cells can be re-used several times.

Data evaluation of single-molecule measurements

The fluorescence signal detected in a zmw of a SMRT cell was stored in a trace file (suffix .trc). Fluorescence intensity was recorded with a frequency of 75 Hz (frames/s) and 8 bit resolution to generate a time course. Raw data were read using the R-package *pbh5* from Pacific Biosciences.¹³ For data evaluation, the fluorescence profile was first smoothed with a median filter of width. The smoothed profile was segmented into plateaus using the Bioconductor package GLAD. We classified a zmw as containing one probe molecule if a) its histogram had two maxima and b) it only had two different intensity levels. An intensity change of larger than 5 intensity units was considered to be a change of binding state (binding/decay). Additionally, we merged a segment with its neighbor if it lasted less than 100 frames. For calculation of kinetic constants, we used all zmw's satisfying the above criteria and pooled together pulse widths (PWs) and interpulse durations (IPDs), respectively, found in individual zmw's. The first and last PW or IPD of each zmw was omitted because of truncation. From the pooled PWs (IPDs) we derived a first (second) order rate constant k_{off} (k_{on}) by running a negative exponential fit. This approach assumes that PWs and IPDs are independent and identically distributed

in all used zmw's, and represent an ensemble average instead of using a long time average of PWs and IPDs obtained from a single zmw. At too low analyte concentration, a second measurement at higher concentration using the same chip is needed in order to determine zmw's containing only one immobilized probe.

RESULTS AND DISCUSSION

Absorbance and fluorescence measurements

Figure 2 shows absorbance and fluorescence spectra of CY3-7, DY547-7 and the dye DY-547, respectively. The absorbance spectra (Figure 2a) of the dye-oligonucleotide conjugates do not show significant band shift and broadening compared with DY-547 indicating the lack of strong interactions.

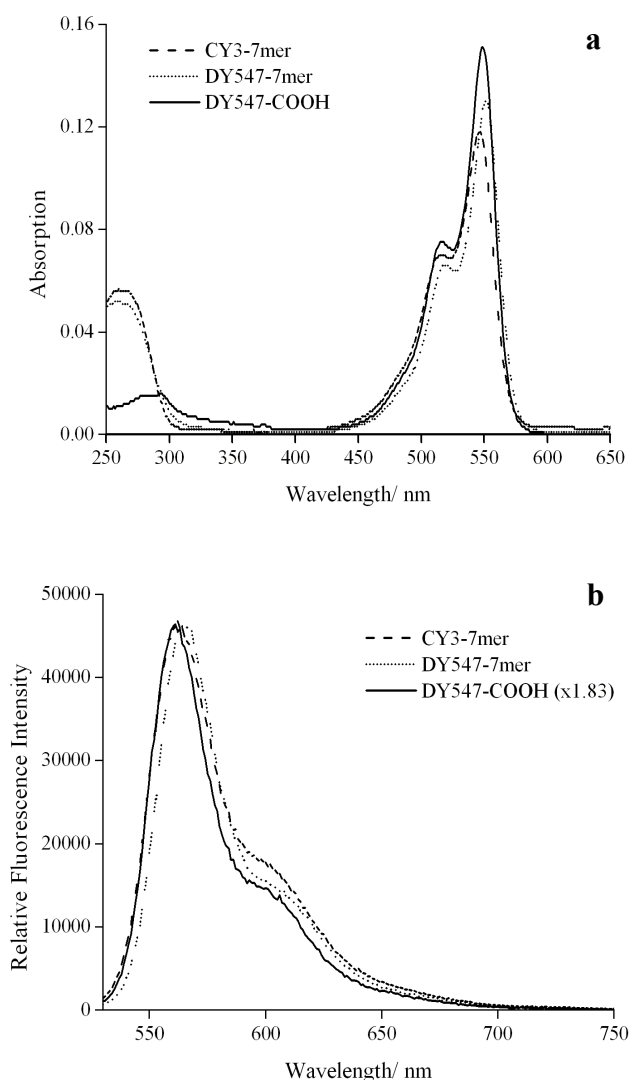


Figure 2. Absorption (a) and fluorescence (b) spectra of CY3-7, DY547-7, and DY-547 free acid (1 μ M in HBS). Emission was excited at 500 nm (bandwidth 10 nm/10 nm).

The fluorescence spectra of CY3-7, DY547-7 and DY-547 are very similar (Figure 2b). No spectral changes are observed upon tenfold dilution (data not shown). Fluorescence intensities of CY3-7 and DY547-7 are nearly identical but by a factor of 1.8 larger than that of the free dye DY-547. This behavior is attributed to an increase in fluorescence

quantum yield at conjugation to the oligonucleotide caused by a reduction in conformational freedom decreasing the efficiency of photoisomerization as derived from measurements of time-resolved fluorescence and anisotropy decay.^{14,15}

Surface plasmon resonance

Figure 3 shows typical sensograms obtained from kinetic experiments of binding of 7mer, DY547-7 and CY3-7, respectively, to Bio34. For details of data processing see Materials and Methods.

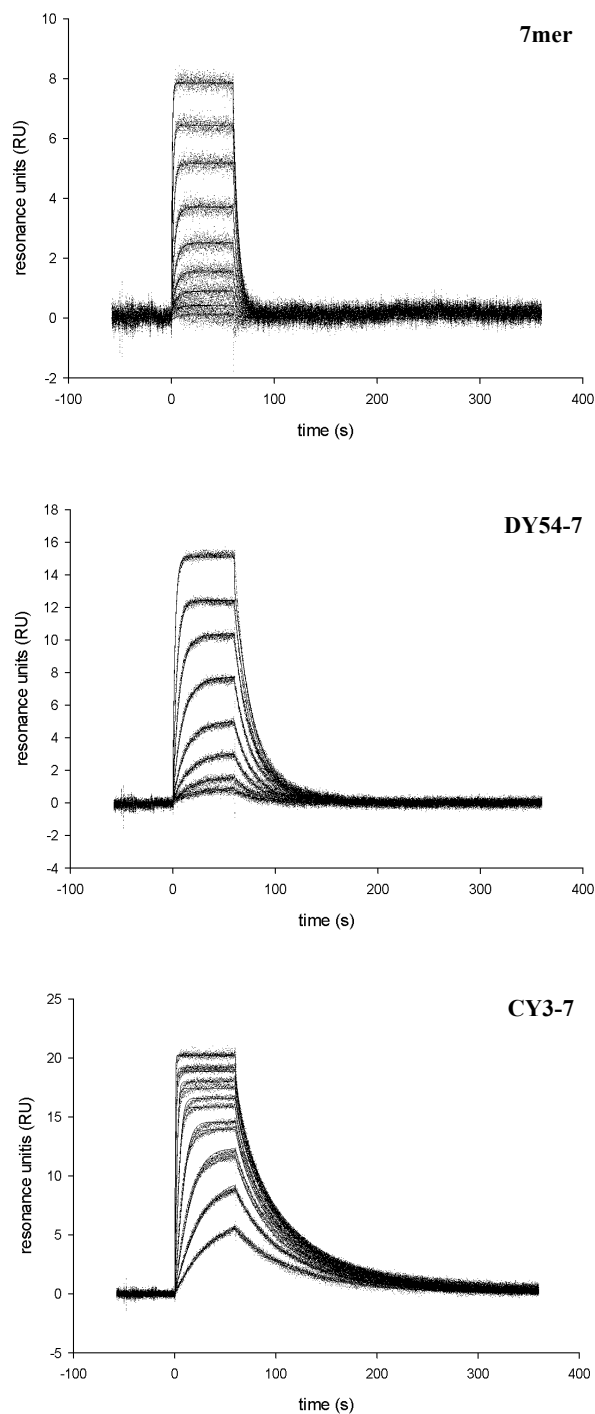


Figure 3. Data from double referenced sensograms of binding of 7mer, DY547-7 and CY3-7, respectively, to Bio34 are shown as point clouds. The fitted data are depicted as solid lines.

The determined k_{on} and k_{off} as well as the calculated K_d values are listed in Table 2. Interestingly, the binding strength increases in the order 7mer, DY547-7 to CY3-7. Thus, despite of the dye DY-547 introducing a net negative charge, the hybrid with DY547-7 is more stable compared to the unlabeled 7mer oligonucleotide. This effect is even more pronounced in the case of CY3-7 bearing a positively charged CY3 dye. The affinity to Bio34 is more than one order of magnitude higher compared to the 7mer.

Table 2. Overview of the kinetic constants k_{on} , k_{off} and K_d for hybridization of 7mer, CY3-7 and DY547-7 to Bio34, respectively, measured by SPR and SMRT. Errors were calculated from a different number of measurements (No.) performed using CM5 and CMD chips.

Analyte	SPR				Single Molecule			
	$k_{\text{on}}/10^5 \text{ M}^{-1} \text{ s}^{-1}$	$k_{\text{off}}/\text{s}^{-1}$	K_d/nM	No.	$k_{\text{on}}/10^5 \text{ M}^{-1} \text{ s}^{-1}$	$k_{\text{off}}/\text{s}^{-1}$	K_d/nM	No.
7mer	4.2 ± 1.4	0.22 ± 0.04	560 ± 60	2	-	-	-	-
Cy3-7	20 ± 3.1	0.04 ± 0.008	20 ± 4	4	24 ± 5	0.08 ± 0.01	36 ± 8	4
DY547-7	3.2 ± 0.04	0.06 ± 0.01	200 ± 35	3	6 ± 1	0.16 ± 0.02	260 ± 56	4

Single-molecule real-time measurements

We investigated the hybridization of two CY3 and DY-547 dye labeled 7mer target oligonucleotides (CY3-7, DY547-7), respectively, to a 34mer oligonucleotide probe (Bio34) immobilized at the bottom of a zero-mode waveguide nanostructure array. The trace files in Figures 4a-c show typical time courses of fluorescence emitted from a hybridized oligonucleotide-dye conjugate for the two oligonucleotides. Diffusing conjugates in solution only contribute to background fluorescence. Their motion through the observation volume lasts a few microseconds that is below the time resolution of the detector (0.13 ms). In case more than one probe molecule Bio34 is immobilized in a zmw a superposition of target binding is observed (Figure 4c).

a

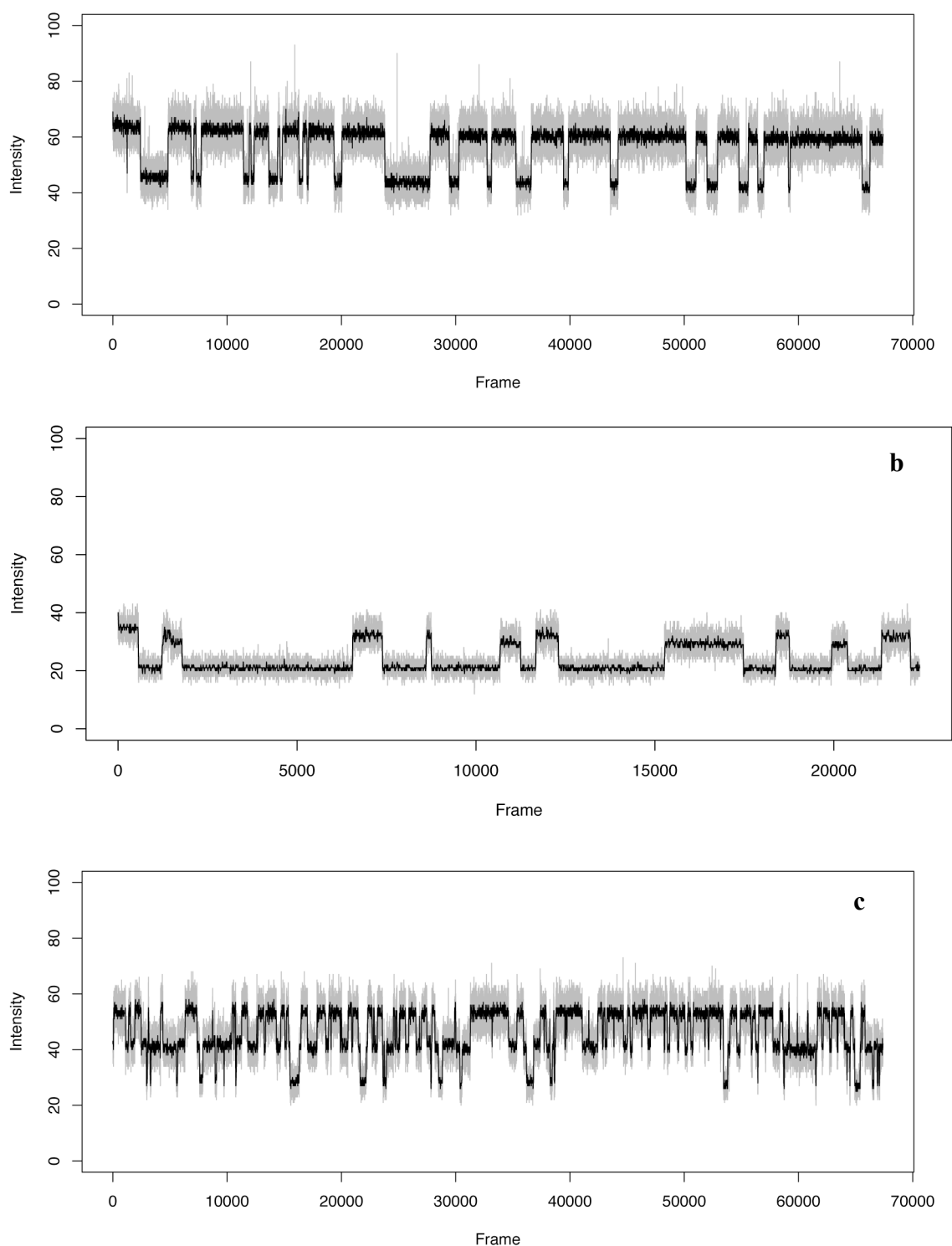


Figure 4. Trace files of fluorescence pulses emitted by a) a single CY3-7 molecule (40 nM, measuring time 15 min), b) a single DY547-7 molecule (40 nM, 5 min), c) CY3-7 in a zmw containing two immobilized probe molecules (40 nM, 15 min) showing a superposition of pulses.

For data evaluation, only those trace files were used originating from zmw's with only one probe molecule immobilized. For details of data processing see Materials and Methods. An exponential function was fitted to the histogram of pulse widths and interpulse durations, respectively, added up over all trace files from those zmw's meeting the filtering criteria of the software. Figures 5a and b show fit results for both oligonucleotides.

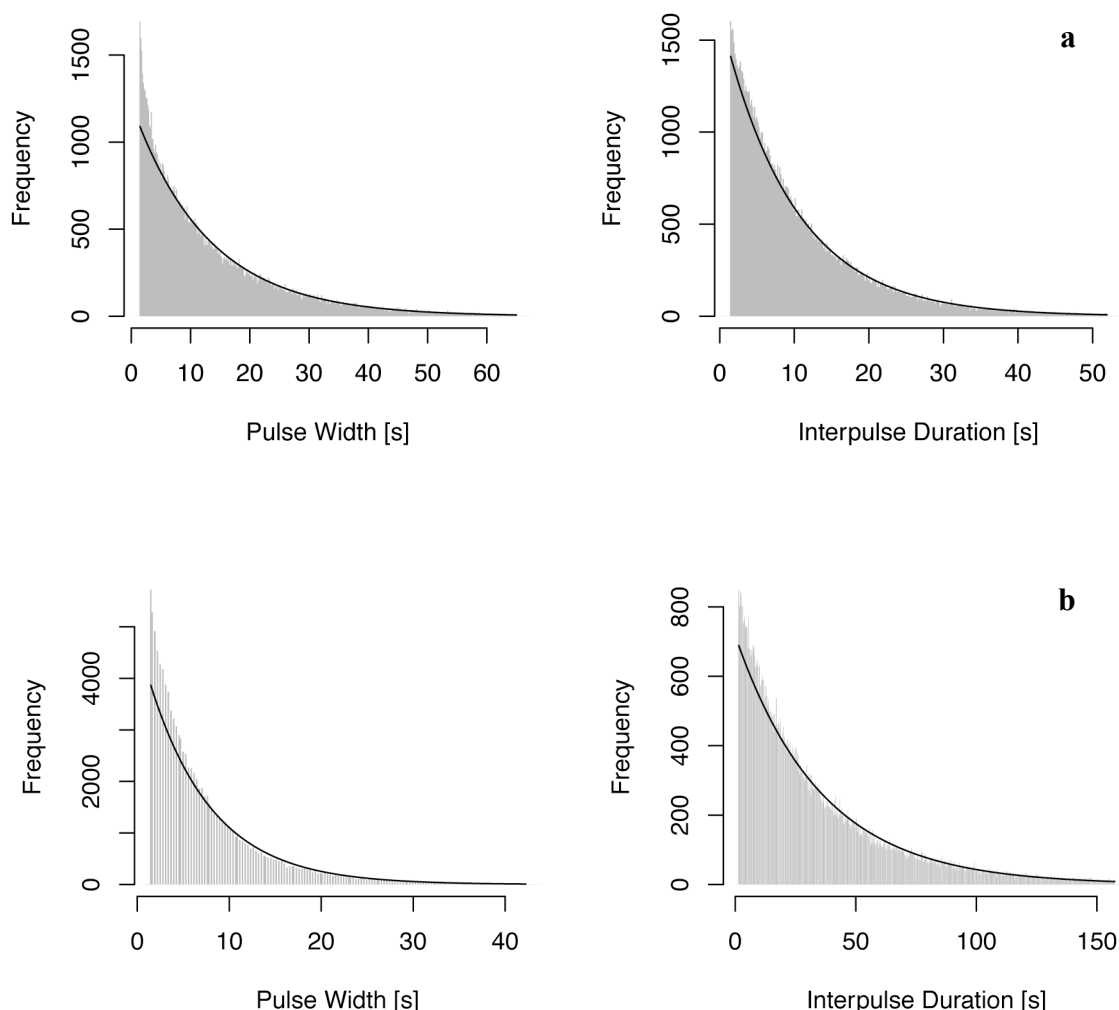


Figure 5. Monoexponential fits of pulse widths and interpulse durations, respectively, measured for a) CY3-7, b) DY547-7.

Kinetic constants are summarized in Table 2. The association rate constant k_{on} was calculated from the time period between two pulses ($\text{IPD} = 1/(k_{\text{on}} \cdot c)$). k_{on} is of second order and depends on the oligonucleotide concentration c . We observe an about two times larger k_{on} for CY3-7 compared to DY547-7. We attribute this to the positive charge in the dye CY3 being attracted by the strongly negatively charged probe. The dissociation rate constant k_{off} is of first order and calculated from the reciprocal pulse width ($\text{PW} = 1/k_{\text{off}}$). k_{off} for CY3-7 is 6 times smaller than for DY547-7 indicating a larger stabilization of the duplex by the positively charged CY3 dye.¹⁵ This results in binding constants K_d ($k_{\text{off}}/k_{\text{on}}$) of 36 nM for CY3-7 and 260 nM for DY547-7, respectively.

For comparison, we measured CY3-7 under the same conditions on an RS sequencer at Pacific Biosciences in Menlo Park. The kinetic constants observed ($k_{\text{off}} = 0.07 \text{ s}^{-1}$, $k_{\text{on}} = 1.9 \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$, $K_d = 37 \text{ nM}$) agree very well with measurements on our RS. The results are in accordance with literature data of comparable systems. However, directly comparable data are rare.^{17,18}

Comparison of SPR and SMRT measurements

Despite the large methodological differences between the two approaches, results observed for hybridization of short oligonucleotides in the SMRT experiments show a good agreement with SPR data (Table 2). Rate constants for association and dissociation and the dissociation constant for a given target oligonucleotide agree within a factor of two. Consistently larger rate constants are observed in SMRT experiments. A reason for this may be the different surface

chemistry applied in the SPR flow cell and the SMRT chip. Although the oligonucleotide probe is immobilized via neutravidin (in the CMD chip), the negatively charged 3D dextrane matrix in the flow cell may provide different conditions for hybridization. To investigate the influence of the surface chemistry on hybridisation we plan to modify a SPR chip surface such that it is comparable to the conditions in a SMRT cell.

An advantage of SMRT is the possibility to investigate interactions with high dissociation constant K_d in the μM and mM range. For SPR, in order to observe k_{off} , the time course of the decay of a probe - analyte complex is evaluated. In case of a too fast reaction, a decay curve does not have enough data points and/or curvature to obtain a reasonable fit. For SMRT, the smallest rate constant obtained is determined by the (mean) pulse width. Since pulse widths as short as only some hundred milliseconds can be detected, analytes having a high dissociation rate constant can be investigated with k_{off} ranging between 1 to 10 s^{-1} . In contrast, k_{off} measured by SPR is limited to about 0.6 s^{-1} .

SMRT technology as a fluorescence based method requires the use of a fluorescent dye which often causes a number of problems. The dye may not only interact with the target it is attached to as shown above. Organic dyes also interfere with their inherent instability by intermittent fluorescence (blinking) leading to intensity changes in the millisecond timescale and photodegradation especially in the presence of oxygen. The use of a triplet quencher (e.g. Trolox) and removing oxygen (e.g. enzymatically, PCD/PCA, see Materials and Methods) is therefore essential. However, even under such conditions, measurements are challenging. For a CY5-labeled 7mer oligonucleotide we do not observe evaluable pulses, but a high number of short spikes instead. Long wavelength absorbing dyes including CY5 are known to be prone to blinking.

The potential of SMRT to study molecular interactions will be investigated in more detail on hybridization of very short oligonucleotides and other systems to explore the options of the new technology.

CONCLUSIONS

In summary, we succeeded in measuring kinetic constants of hybridization of short oligonucleotides with single molecule resolution in an RS sequencer. We find good agreement of results observed by using SPR and SMRT. The single-molecule method seems to give larger values for the rate constants. However, considering the limited dataset, a final conclusion with respect to systematic deviations of results cannot be drawn at this pilot stage.

Moreover, we observed a pronounced dye effect that strongly influences the association constant k_{on} in the case of CY3 dye bearing a positive charge. Irrespective of the charge, the labeling dye stabilizes the DNA hybrid resulting in a decrease of the dissociation constant by a factor of 2.5 and 25 for DY547-7 and CY3-7, respectively, compared to the unlabeled 7mer. Since the dye is usually considered not to interfere with the measurement of an interaction, our results suggest that the stabilizing effect must be taken into account upon selection of a dye for labeling purposes.

ACKNOWLEDGMENTS

We wish to thank Dr. Jonas Korch, Dr. Khai Luong and Gerrit Kuhn from Pacific Biosciences (Menlo Park, CA) for their generous support, helpful discussions, and day and night technical assistance. We thank Dr. Markus Schmid from Microsynth (Balgach, Switzerland) for his support and helpful discussions in all matters concerning oligonucleotides.

REFERENCES

- [1] Levene, M.J., Korch, J., Turner, S. W., Foquet, M., Craighead, H. G. and Webb, W. W., "Zero-mode waveguides for single-molecule analysis at high concentrations," *Science* 299 (5607), 682-686 (2003).
- [2] Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., Peluso, P., Rank, D., Baybayan, P., Bettman, B., Bibillo, A., Bjornson, K., Chaudhuri, B., Christians, F., Cicero, R., Clark, S., Dalal, R., Dewinter, A., Dixon, J., Foquet, M., Gaertner, A., Hardenbol, P., Heiner, C., Hester, K., Holden, D., Kearns, G., Kong, X., Kuse, R., Lacroix, Y., Lin, S., Lundquist, P., Ma, C., Marks, P., Maxham, M., Murphy, D., Park, I., Pham, T., Phillips, M., Roy, J., Sebra, R., Shen, G., Sorenson, J., Tomaney, A., Travers, K., Trulson, M., Vieceli, J., Wegener, J., Wu, D., Yang, A., Zaccarin, D., Zhao, P., Zhong, F., Korch, J. and Turner, S., "Real-time DNA sequencing from single polymerase molecules," *Science* 323 (5910), 133-138 (2009).

- [3] Hoa, X. D., Kirk, A. G. and Tabrizian, M., "Towards integrated and sensitive surface plasmon resonance biosensors: A review of recent progress," *Biosensors and Bioelectronics* 23 (2), 151-160 (2007).
- [4] Homola, J., Yee, S. S. and Gauglitz, G., "Surface plasmon resonance sensors: review," *Sensors and Actuators B: Chemical* 54 (1-2), 3-15 (1999).
- [5] Korlach, J., Bjornson, K. P., Chaudhuri, B. P., Cicero, R. L., Flusberg, B. A., Gray, J. J., Holden, D., Saxena, R., Wegener, J. and Turner, S. W. "Real-time DNA sequencing from single polymerase molecules," *Methods in Enzymology* 472, 431-455 (2010).
- [6] Korlach, J., Marks, P. J., Cicero, R. L., Gray, J. J., Murphy, D. L., Roitman, D. B., Pham, T. T., Otto, G. A., Foquet, M. and Turner, S. W. "Selective aluminium passivation for targeted immobilization of single DNA polymerase molecules in zero-mode waveguide nanostructures," *PNAS* 105 (4), 1176-1181 (2008).
- [7] Jönsson, U., Fägerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Löfas, S., Persson, B., Roos, H., Rönnerberg, I., "Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology," *Biotechniques* 11 (5), 620-627 (1991).
- [8] Malmqvist, M., "Biospecific interaction analysis using biosensor technology," *Nature* 361 (6408), 186-187 (1993).
- [9] Myszka, D. G., "Improving biosensor analysis," *J. Mol. Recognit.* 12 (5), 279-284 (1999).
- [10] Rich, R.L., Myszka, D. G., "Advances in surface plasmon resonance biosensor analysis," *Curr. Opin. Biotechnol.* 11 (1), 54-61 (2000).
- [11] Zheng, Q., Jokusch, S., Zhou, Z., Altman, R. B., Warren, J. D., Turro, N. J. and Blanchard, S. C. "On the mechanisms of cyanine fluorophore photostabilization," *J. Phys. Chem. Lett.* 3 (16), 2200-2203 (2012).
- [12] Aitken, C. E., Marshall, R. A. and Puglisi, J. D. "An oxygen scavenging system for improvement of dye stability in single-molecule fluorescence experiments," *Biophysical Journal* 94 (5), 1826-1835 (2008).
- [13] <https://github.com/PacificBiosciences/R-pbh5>
- [14] Sanborn, M. E., Connolly, B. K., Gurunathan, K., Levitus, M., "Fluorescence Properties and Photophysics of the Sulfoindocyanine Cy3 Linked Covalently to DNA," *J. Phys. Chem. B* 111, 11064-11074 (2007).
- [15] Harvey, B. H., Levitus, M., "Nucleobase-Specific Enhancement of Cy3 Fluorescence," *J Fluoresc* 19, 443-448 (2009).
- [16] Henry, M. R., Stevens, P. W., Sun, J. and Kelso, D. M. "Real-time measurements of DNA hybridization on microparticles with fluorescence resonance energy transfer," *Anal. Biochem.* 276 (2), 204-214 (1999).
- [17] Jungmann, R., Steinhauer, C., Scheible, M., Kuzyk, A., Tinnefeld, P. and Simmel, F. C. "Single-molecule kinetics and super-resolution microscopy by fluorescence imaging of transient binding on DNA origami," *Nano Lett.* 10 (11), 4756-4761 (2010).